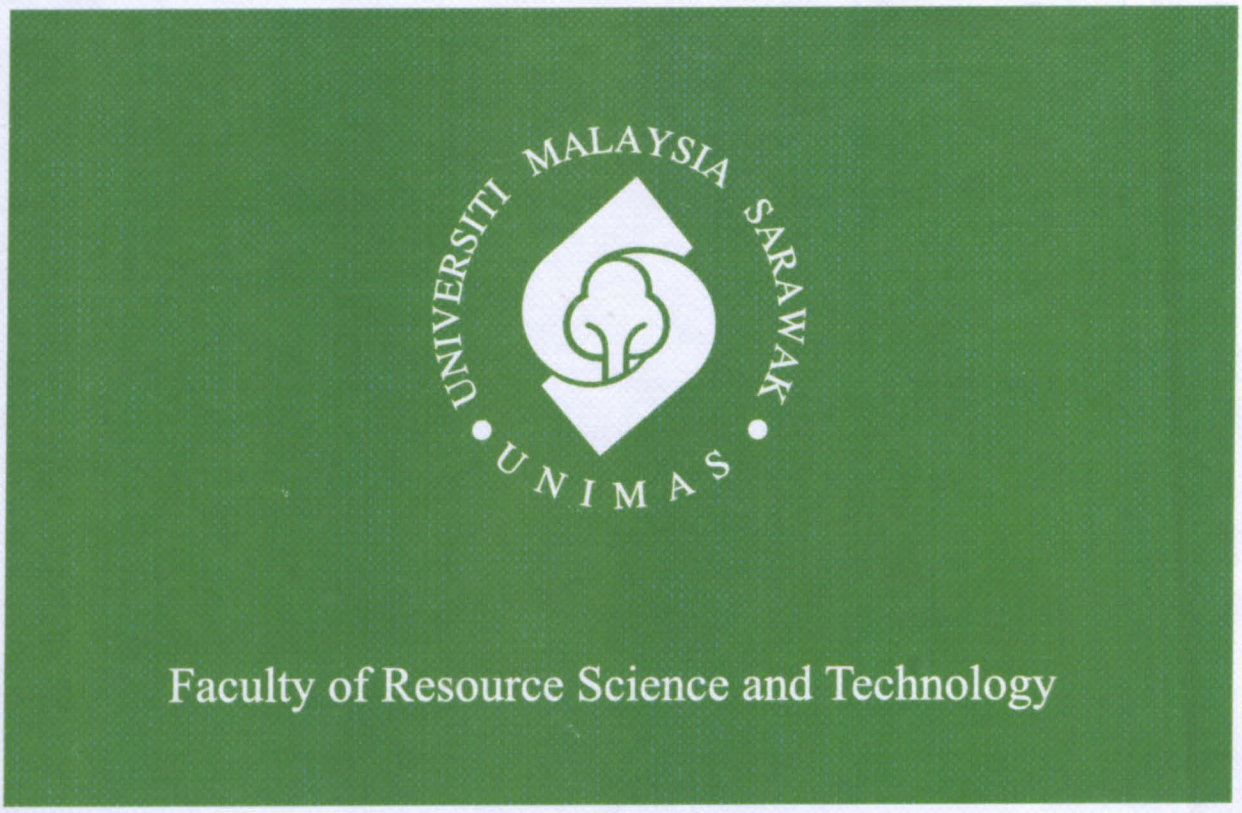

Isolation and Characterization of Heterocyclic Hydrocarbon Degrading Bacteria from Industrial Waste Water

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(50181)

Bachelor of Science with Honours
(Resource Biotechnology)
2017

Isolation and Characterization of Heterocyclic Hydrocarbon Degrading Bacteria from Industrial Waste Water

Ummuhatul Jannah bt Yatim (50181)

A final year project report submitted in partial fulfilment of the
Final Year Project 2 (STF 3015) course

Supervisor: Dr. Azham bin Zulkharnain

WS47-Resource Biotechnology
Department of Molecular Biology

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Universiti Malaysia Sarawak
2017

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LIST OF ABBREVIATION

DNA	Deoxyribonucleic acid
SDS	Sodium Dodecyl Sulfate
MSM	Mineral Salt Medium
PAHs	Polycyclic Aromatic Hydrocarbons
$(NH_4)_2SO_4$	Ammonium sulfate
$CaCl_2.2H_2O$	Calcium chloride dehydrate
Na_2HPO_4	Disodium phosphate
$FeSO_4.7H_2O$	Iron (II) sulfate heptahydrate
$MnSO_4.7H_2O$	Manganese sulfate heptahyrate
$MgSO_4.7H_2O$	Magnesium sulfate heptahyrate
KH_2PO_4	Potassium dihydrogen phosphate
$Na_2MoO_4.H_2O$	Sodium molybdate dehydrate

Isolation and Characterization of Heterocyclic Hydrocarbon Degrading Bacteria from Industrial Waste Water

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ABSTRACT

Urbanization is one of the major sources of water pollution. It has been identified that one of the major component in the contaminant are heterocyclic hydrocarbon compounds. It was shown that these compounds are hazardous and remain in the environment as resistant pollutants for long period of time. The objective of this study is to isolate, identify, characterize and assess degradation rate of heterocyclic hydrocarbon degrading bacteria from industrial waste water. Two bacteria strains that possess the ability to utilize heterocyclic hydrocarbon compounds specifically carbazole as sole carbon source was successfully isolated from the industrial wastewater sample collected in Demak Laut Industrial Park Sarawak area. The bacteria strains labelled as D1 and B2 were isolated using enrichment cultures and double layer agar method. Then the isolates DNA were extracted and amplified using Polymerase Chain Reaction (PCR). Restriction Fragment Length Polymorphism (RFLP) was used to confirmed that both isolates were from distinct species. The isolates were sequenced and isolate D1 was identified as *Achromobacter* sp. based on the 16S rRNA sequence similarity, while isolate B2 was failed to be sequenced. Both isolates also possess the ability to grow on other heterocyclic hydrocarbons, dibenzofuran (DBF) and dibenzothiophene (DBT) as sole carbon source. The ability of the bacteria to degrade carbazole was measured using Gas Chromatography Mass Spectrophotometry (GC-MS) and after 12 days the isolates D1 and B2 was able to degrade 71.68% and 60.06% of the carbazole respectively. Bacteria isolated from this study may be beneficial as one of the potential candidate in bioremediation.

Keywords: Heterocyclic hydrocarbon, carbazole, degrading bacteria

ABSTRAK

Pembandaran adalah salah satu sumber utama pencemaran air. Ia telah dikenal pasti bahawa salah satu komponen utama dalam bahan cemar tersebut adalah sebatian heterosiklik hidrokarbon. Ia menunjukkan bahawa sebatian ini adalah berbahaya dan boleh kekal dalam alam sekitar untuk tempoh masa yang panjang. Objektif kajian ini adalah untuk mengasingkan, mengenal pasti, mencirikan dan menilai kadar degradasi bakteria pengurai heterosiklik hidrokarbon daripada air sisa perindustrian.,Dua bakteria strain yang dapat menggunakan sebatian heterosiklik hidrokarbon khususnya karbazol sebagai sumber karbon tunggal telah berjaya diasingkan daripada sampel air sisa perindustrian yang diambil di kawasan Taman Perindustrian Demak Laut Sarawak. Bakteria straindilabelkan sebagai D1 dan B2 telah diasingkan dengan menggunakan kaedah pengkulturan dan kaedah dua lapisan agar. Kemudian DNA bakteria tersebut diekstrak dan diperbanyakkan menggunakan Reaksi Rantaian Polymerase (PCR). Restriction Fragment Length Polymorphism (RFLP) telah digunakan untuk mengesahkan perbezaan spesies kedua-dua bacteria tersebut. Bakteria D1 telah dikenal pasti sebagai Achromobacter sp. berdasarkan persamaan analisis jujukan16S rRNA, manakala bakteria B2 tidak Berjaya dikenalpasti.kedua-dua strain ini juga dapat tumbuh di atas Media Garam Mineral (MSM) agar dengan sumber heterosiklik hidrokarbon lain,dibenzofuran(DBF) dan dibenzothiophene-(DBT) sebagai sumber karbon tunggal. Keupayaan bakteria untuk mengurai carbazole juga diukur menggunakan Gas Chromatography Mass spektrofotometri (GC-MS) dan selepas 12 hari strain D1 dan B2 ini masing-masing dapat mergurai 71.68% dan 60.06% % daripada carbazole itu. Bakteria yang diasingkan dari kajian ini mungkin dapat dimanfaatkan sebgai salah satu calon yang berpotensi digunakan dalam pemuliharaan alam sekitar.

Kata kunci: Heterosiklik hidrokarbon, karbazol, bakteria pengurai

1.0 INTRODUCTION

Industrial waste water is one of the major sources of water pollution. Initially water pollution was limited to small and localized area (Hanchang, 2009). However now after the industrial revolution and rapid development of various industries, water is use as cooling purpose, a raw material and as a means of production or process water causing water to become an essential waste product. Different industries will produce different type of waste water, for example, the petrochemical industry will produce a lot of mineral oils and phenol, while pulp and paper industry produce dioxins and chlorine organics as well as organic waste and suspended solids. Some industry that have good management may conduct treatment before releasing the waste water into environment and some with poor management may discharge untreated industrial waste water to environment such as river and sea.

One of the compounds that can be found in contaminate water are heterocyclic hydrocarbon compound. This aromatic compound is a cyclic compound that contains at least two or more carbon ring. According to Gai *et al.* (2007), this compound possesses toxic and mutagenic activities therefore making it hazardous and possible carcinogenic. It may also remain in environment as a resistant pollutant. This will not only affect the environment, but also harmful to human who may consume the water as drinking water or food such as fish from this contaminated water. For example, according to Pasternak *et al.* (2012), indole can be toxic to the aquatic organisms and also can cause irritation and damage to the human skin. Some of heterocyclic hydrocarbon compound that have their degradation product detected in petroleum and industrial waste water contaminated site such as seawater, groundwater, soil and sediment are biphenyl, carbazole, dibenzofuran, dibenzothiophene, and fluorine.

However, there are ways that can be used to overcome this problem. One of them is bioremediation. According to Seo *et al.* (2009), in bioremediation, bacteria can be used to neutralize the organic pollutant into harmless metabolite or mineralize the pollutants into carbon dioxide and water. This method is efficient and cost-effective because it relies on the natural ability of the bacteria. Therefore the degrading bacteria play an important role in helping to remove the contamination in the environment. Research about heterocyclic hydrocarbon degrading bacteria may be useful for bioremediation research and contribute in reducing contamination leading to a better environment.

Hence the objectives of this project study are:

1. To isolate the heterocyclic hydrocarbon degrading bacteria from industrial waste water
2. To identify the isolated heterocyclic hydrocarbon degrading bacteria
3. To characterize and to measure the degradation ability of the isolated heterocyclic hydrocarbon degrading bacteria

2.0 LITERATURE REVIEW

2.1 Bioremediation

In bioremediation, "Bio" stands for life, while "remediate" means to solve a problem. Therefore, bioremediation can be defined as the use of biological organisms to solve an environment problem. It is also known as a process where the living organisms is use to degrade the organic waste into less toxic form which is below the level of concentration limit establish by the authorities (Mueller *et al.*, 1996).

There are two types of remediation techniques, ex-situ and in-situ. Ex-situ is done by removing the contaminated water or soil and treating it outside the source, this technique include all that are applied to groundwater and soil which has been removed from the site via pumping or excavation (Vidali, 2001). While in in-situ, the techniques are applied to soil and groundwater at the site with minimal disturbance or in other word the treatment is conducted within the contaminated area (Zeyaullah *et al.*, 2009).

Both of these techniques can be used in bioremediation of polycyclic aromatic hydrocarbons (PAH) contaminated soils, sediment, and water (Bamforth & Singleton, 2005). Ex-situ usually used for smaller projects, primarily because larger excavation or movement of soil can be more detrimental by destroying the pre-establish horizons in the soil. Some of the method use in ex-situ includes composting, bioreactors, land-farming and bio-piles. For in-situ techniques, the methods include bio-stimulation, bioleaching, bio-sorption, and bioventing. In-situ is more preferred compare to ex-situ due to the cost efficient and minimal invasive to the soil structure.

2.2 Degrading Bacteria

Bacteria are single cell microscopic and widely diverse organisms. It can live in diverse environment such as soil, ocean and river. Therefore it can be very useful bioremediation. In right condition, bacteria most likely can break any given substrate. In previous study algae, bacteria and fungi that are capable of degrading Polycyclic aromatic hydrocarbons (PAHs) have been isolated and characterized (Cerniglia, 1992) and most of them are isolated from contaminated soil or sediments. Some of the commonly studied PAH-degrading bacteria are *Rhodococcus* spp. (Dean-Ross *et al.*, 2002), *Pseudomonas aeruginosa* (Romero *et al.*, 1998), *Mycobacterium* spp. (Rehmann *et al.*, 1998), *Pseudomons fluoresens*, and *Haemophilus* spp. (Yuan *et al.*, 2002).

2.3 Heterocyclic Hydrocarbon

Hydrocarbons are an organic compound made from hydrogen and carbon atoms. It can be found in many places. One of the examples is fossil fuel, it is stored deep underground but are brought up to the surface to be transformed and utilized, primarily as an energy source. Unfortunately, majority of pollution currently comes from these byproducts in the form Polycyclic Aromatic Hydrocarbons (PAHs). Polycyclic Aromatic Hydrocarbons (PAHs) are organic compounds that contain two or more fused benzene rings which are arranged in various structural configurations (Haritash & Kaushik, 2009). They can persist in the environment as contaminants that are toxic, mutagenic and carcinogenic. PAHs can occurs naturally or by human activities. It formed from coal tars, petroleum residues, motor vehicle exhaust, tobacco smoke, burning of fossil fuel, wood, garbage, used lubricating oil and oil filters (Haritash & Kaushik, 2006). In these sources, heterocyclic aromatic compounds (HACs) are present along with PAHs (Warshawsky, 1992). HACs are compound that contain one or more nitrogen, sulfur, or oxygen atoms.

Based on Bamforth and Singleton (2005), as shown in the Figure 2.1 below, in the aerobic metabolism of PAHs by microorganisms, there are three fundamentally different mechanisms. These mechanisms are bacteria degradation, ligninolytic fungal degradation and non-ligninolytic fungal and bacterial degradation. These mechanisms basically start with the oxidation of the aromatic ring, followed by the systematic breakdown of the compound to PAH metabolites and carbon dioxide.

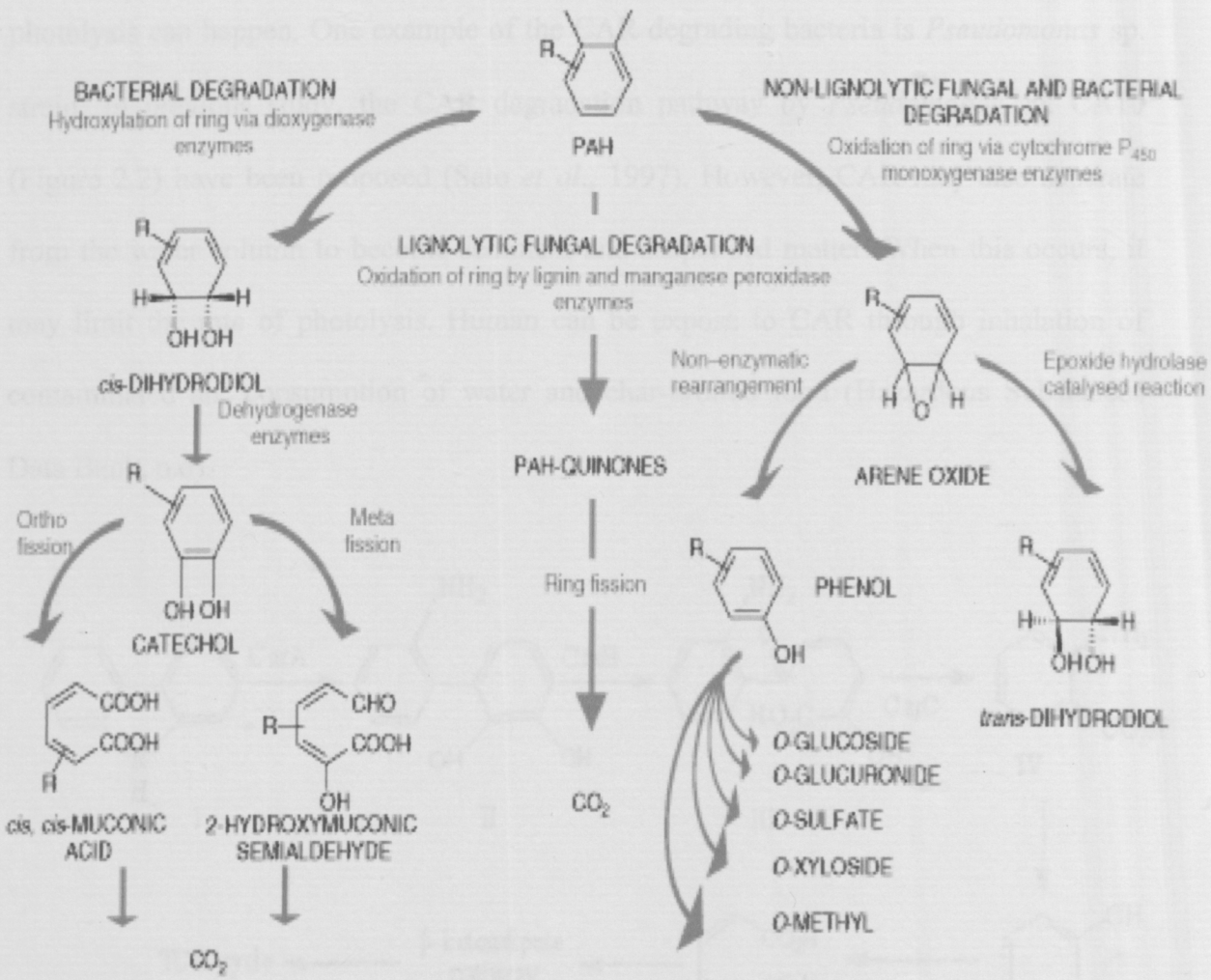

Figure2.1: The three main pathways for polycyclic aromatic hydrocarbon degradation by fungi and bacteria (Bamforth & Singleton, 2005).

2.3.1 Carbazole (CAR)

Carbazole (CAR) is a five membered nitrogen containing ring that has two six membered benzene ring on both side. It is also known as an N-heterocyclic hydrocarbon. It can be found in creosote, crude oil, shale oil and also used as a feedstock for the manufacture of dyes plastics and medicines (Singh *et al.*, 2011). If CAR is released to water, with specific degrading bacteria and sufficient sunlight, biodegradation and photolysis can happen. One example of the CAR degrading bacteria is *Pseudomonas* sp. strain. In previous study, the CAR degradation pathway by *Pseudomonas* sp. CA10 (Figure 2.2) have been proposed (Sato *et al.*, 1997). However, CAR may also separate from the water column to become sediment and suspended matter. When this occurs, it may limit the rate of photolysis. Human can be expose to CAR through inhalation of contaminated air, consumption of water and char-broiled food (Hazardous Substances Data Bank, n.d).

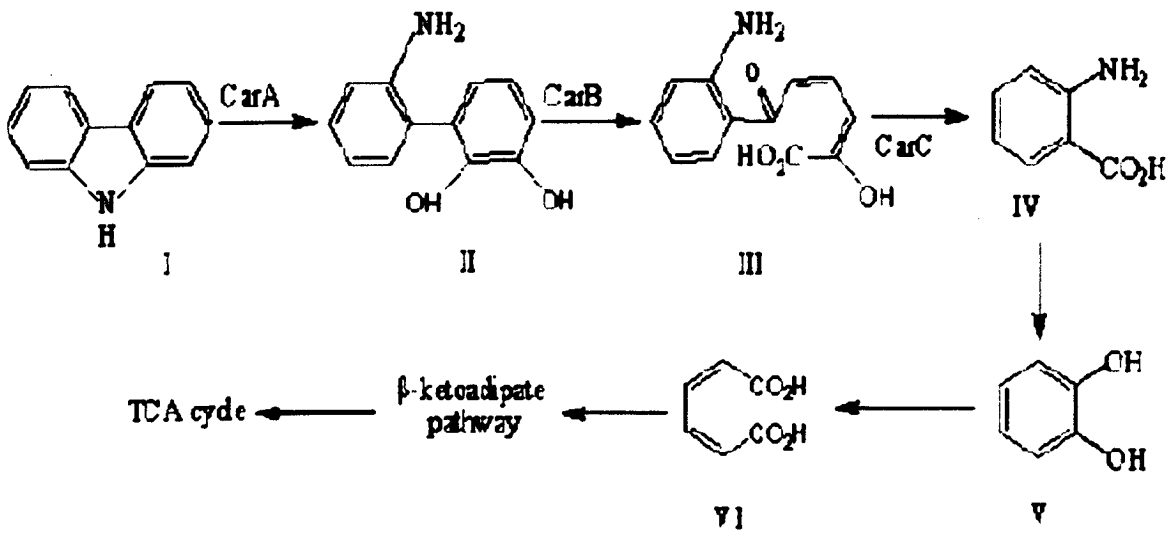

Figure 2.2: Degradation pathway of carbazole by *Pseudomonas* sp. strain CA10. CarA, carbazole 1,9a-dioxygenase; CarB, 2'-aminobiphenyl-2,3-diol-1,2-dioxygenase; CarC, 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoic acid hydrolase; I, Carbazole; II, 2'-aminobiphenyl-2,3-diol; III, 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoic acid (meta-cleavage compound); IV, Anthranilic acid; V, Catechol; VI, cis,cis-muconic acid (Sato *et al.*, 1997).

3.0 MATERIALS AND METHOD

3.1 Sample collection

The water sample was collected from Demak Laut Industrial Park area (Sarawak), as shown in Figure 3.1 and Figure 3.2 below, specifically near the Lee Ling Timber Product Sdn. Bhd. area.

Figure 3.1: Demak Laut Industrial Park area (https://www.google.com/maps)

Figure 3.2: Water sample in Lee Ling Timber Sdn Bhd area

3.2 Preparation of mineral salt media (MSM)

The mineral salt media (MSM) was prepared with 1 L distilled water according to the formulation in Table 3.1 (Atlas, 2010) below. Then the pH was adjusted to be between the ranges of 7.0-7.4.

Table3.1: Mineral Salt Media (MSM) formulation per 1 L of distilled water (Atlas, 2010)

Chemical	Weight, gram (g)
Ammonium sulfate, $(NH_4)_2SO_4$	0.50 g
Calcium chloride dehydrate, $CaCl_2.2H_2O$	0.01 g
Disodium phosphate, Na_2HPO_4	0.00213 g
Iron (II) sulfate heptahydrate, $FeSO_4.7H_2O$	0.005 g
Magnesium sulfate heptahydrate, $MgSO_4.7H_2O$	0.0025 g
Manganese sulfate heptahyrate, $MnSO_4.7H_2O$	0.0020 g
Potassium dihydrogen phosphate, KH_2PO_4	1.36 g
Sodium molybdate dihydrate, $Na_2MoO_4.H_2O$	0.0025 g

3.3 Preparation of mineral salt media (MSM) double layer agar supplemented with heterocyclic hydrocarbon

The mineral salt media (MSM) double layer agar was prepared by adding the MSM broth with bacto agar. For the lower layer, 2.25 gram of bacto agar was added to 150 ml of MSM broth in a conical flask. While the upper layer, was prepared by adding 3.75 gram of bacto agar into 250 ml MSM broth in another conical flask. Then both of the conical flasks containing the agar mixture were autoclaved. The MSM agar mixture for the upper layer was supplemented with 0.1% carbazole (CAR) as a sole carbon source. First, the MSM agar mixture without CAR were poured into 20 plates and allowed to solidify. Then the second MSM agar mixture containing the 0.1% CAR was poured onto

the solid MSM agar and allowed to solidify. The step was again repeated to make MSM agar with other substrate, dibenzofuran-(DBF) and dibenzothiophene-(DBT). All the agar then was stored in a refrigerator for future usage.

3.4 Culture enrichment and bacteria isolation

For first enrichment, four test tubes with screw cap containing 10 ml Mineral Salt Media (MSM) were prepared. Two of the tubes were added with 0.1% carbazole (CAR), while the other two tubes added with 0.01% CAR. Each one of the tubes containing 0.1% CAR and 0.01% CAR will act as a control while the other two tubes were added with 1 ml of water sample respectively. The tubes then was left on a shaker at room temperature until colour changes was observed. Table 3.2 below show the summary of tubes label and contains.

Table3.2: Tubes label and contains for first enrichment

Tubes	Contains
A	Control (MSM + 0.1% CAR)
B	Control (MSM + 0.01% CAR)
C	MSM + 0.1 % CAR + water sample
D	MSM + 0.01% CAR + water sample

After first enrichment, the sample were streaked on the MSM double layer agar and 1 ml of sample from tubes C and D were transferred into a new tubes for second enrichment. After a colour change was observed, the sample from second enrichment was again streaked on the MSM double layer agar with 0.1% substrate as sole carbon source. Then the bacteria were subculture to obtain pure colony.

3.5 DNA extraction

The DNA extraction method was conducted after the bacteria growth in the nutrient broth for 24 hours. 1.5ml of fresh colony were obtained and inoculated into a micro centrifuge tube. Then it was centrifuge at 10 000 rpm for 2 minutes to obtain the pellet. The supernatant was discarded and the pellet was suspended with 567 µl TE buffer. 30µl of 10% sodium dideocyl sulphate (SDS) and 3 µl of protease K were added into the mixture and then incubated for 1 hour at 37°C. Then 100µl of 5 M NaCl was added and mixed thoroughly. 80µl of CTAB was added and the mixture was incubated at 65°C for 10 minutes. After that, equal volume of chloroform/isoamyl alcohol was added and the mixture was centrifuge for 4 to 5 minutes at 13 000rpm. The supernatant were transferred into a fresh micro centrifuge tube and exact volume of phenol/chloroform/isoamyl alcohol with exact volume of supernatant was added before the mixture was centrifuge for 5 minutes. Then the supernatant was transferred again into a new micro-centrifuge tube and 0.6 volume of isopropanol was added into the tube and re-centrifuge for another 15 minutes at 10 000rpm. The supernatant was discarded and the pellet was washed with 200µl of 70% ethanol by centrifuge for 15 min at 13 000rpm. Lastly, the supernatant was removed and the pellets were air dried. Then the pellet was re-dissolved using 50 µl TE buffer and stored in -20°C. The DNA extracted was analyse using agarose gel electrophoresis (AGE) for visualization and conformation before proceeding with PCR.

3.6 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction was conducted using the DNA universal primer which were 27F and 1492R (Hirano *et al.*, 2004) as shown in Table 3.3 below. The PCR mixtures were prepared according to formulation in the Table 3.4 with final volume of 50 µl. Then the mixture will be run in the DNA thermal cycler machine with parameter as shown in Table 3.5.

Table 3.3: Polymerase Chain Reaction universal primer (Hirano *et al.*, 2004)

Primers	Sequences
27F	5' AGAGTTTGATCMTGGCTCAG 3'
1492R	5' TACGGCTACCTTGTTACGACTT 3'

Table 3.4: Reaction mixture for Polymerase Chain Reaction

PCR reagents	Volume (µl)
Sterile distilled water (dH_2O)	36
10X PCR buffer	5
2.5 mM dNTPs	4
50 mM $MgCl_2$	1.5
25 mM forward primer (27f)	1
25 mM reversed primer (1492r)	1
DNA template	1
5 U/ µl Taq DNA Polymerase	0.5
Final volume	50.00

Table 3.5: Parameter for Polymerase Chain Reaction

Phase	Temperature and Duration	
Initial denaturation	95°C for 5 minutes	
Denaturation	95°C for 45 seconds	30 cycles
Annealing	51°C for 40 seconds	
Extension	72°C for 1 minutes 30 seconds	

3.7 Restriction Fragment Length Polymorphism (RFLP)

The Restriction Fragment Length Polymorphism (RFLP) was conducted by adding all the mixture according to the formulation in Table 3.6. below. Then the mixture was incubated for approximately 1 hour and 30 minutes at 37°C. Next the RFLP product was analyse using AGE method and viewed under the UV light.

Table 3.6: Reaction Mixture for RFLP

RE reagents	Amount (µl)
10X RE buffer	1
Restriction Enzyme (Hind III)	0.5
DNA templates	3
ddH_2O	5.5
Total	10

3.8 Agarose Gel Electrophoresis (AGE)

For visualization, Agarose Gel Electrophoresis (AGE) was conducted after DNA extraction, Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP). AGE was prepared by adding 0.5 gram of agarose powder with 50 ml distilled water. This mixture was heated in microwave for 40 seconds and then added with 1ml of 50x TAE buffer after the mixture cooled. Then the mixtures were poured on an agarose gel electrophoresis tray with comb and left approximately 20 minutes until it solidify. 5 µl of the sample product (DNA extraction, Polymerase Chain Reaction product or RFLP product) were loaded into the gel slots. The gel was run for 25 minutes at 90 V and viewed under the ultraviolet (UV) light after soaking the gel in the Ethidium Bromide (EtBr) for 2-3 minutes. After confirmation of PCR and RFLP product the product was send to the First BASE Laboratories Sdn Bhd for sequence analysis. Then the sequence data was further analyse using MEGA software and BLAST from NCBI.

3.9 Growth test with different heterocyclic hydrocarbon substrate

Both isolated bacteria was streaked on MSM double layer agar with two different substrate which was dibenzofuran (DBF) and dibenzothiophene (DBT), to test either the isolated bacteria able to also degrade different heterocyclic hydrocarbon other than carbazole (CAR).